I am still impressed that the textbook language of organic chemistry is so successful in explaining the reactions that we find in nature. This might not have been anticipated because we find that biological reactions are invariably stereospecific whereas nonbiological reactions are invariably not. This realization came to light about 150 years ago when Pasteur found that only half of chemically prepared tartarate was fermentable contrary to chemically identical tartarate from grapes. Only the natural product rotated the plane of polarized light. A new geometry had to be found for carbon to explain this. It could not be planar. As understood by Ogston in 1948 the stereospecificity of biological reactions derives from the chiral properties of the active sites of the enzymes that catalyze them. (Pasteur realized that only a chiral reagent would be able to distinguish between substrates that were not superimposable on their mirror images. He spent much effort searching for a natural force that might have caused chirality in the first place. It remains an unsolved problem.)

When I began studying simple enzymatic reactions at carbon centers in 1955, I was not at all sure what I might run into. Organic chemists were acquiring evidence for stable ion pair intermediates in carbonium and carbanion rearrangements in solution that were completely stereospecific. Would the stereospecificity of enzymatic reactions turn out to be explained in terms of a physical or chemical role of the enzyme? If this seems like an overstatement it may be recalled that in 1955 there were no examples to cite in which an enzyme could be analyzed to be acting as a base to abstract a proton from –CH\(_2\) to a carbonyl. However, there were well known enzymes available such as the aldolases and aldose-ketose isomerases, the mechanisms of which had not been analyzed. My hope then was to use a stereochemical approach to the study of enzyme reaction chemistry as my first research problem.

Fortunately scintillation counters were becoming available in 1955. I was somewhat ahead of the game because of the hobby of Seymour Lipsky, an M.D. in the Department of Medicine who consulted for the New Haven-based Technical Measurement Co. and had one of their first commercial counters. Tritium, especially T-water, was also becoming available, making it unnecessary for me to repair the Rittenberg model mass spectrometer that Henry Hoberman had left to the Yale Department of Biochemistry.

The first demonstration of an enzyme acting as a base was probably our observation in 1955 that muscle aldolase catalyzed the stereospecific exchange of one of the C-1 hydroxymethyl protons of dihydroxyacetone-P with TOH in the absence of an aldehyde partner. From the stereochemistry of the T-exchanged product compared with that of C-4 of the condensation products one
could conclude that the aldehyde and the proton must approach the stable intermediate from the same direction. Evidence that "proton abstraction" is the result of transfer to a stable position on the enzyme and not to an anionic group on substrates comes from a number of examples in which intermolecular proton transfer could be shown. For example, with fumarase the reacting tritium derived from malate can be rescued from exchange with solvent by carrying out the reaction in the presence of fluorofumarate. In fact the loss of the proton from free enzyme is the slowest step of the reaction cycle.

In 1957 the conversion of fructose-6-P to glucose-6-P in D2O had been reported to occur with little or no transfer of the substrate hydrogen to the neighboring carbon of the product by phosphoglucose isomerase (PGI) (5). The absence of transfer might indicate that the enzyme did not act as a base or that different bases were used for C-1 and C-2. We inadvertently discovered that the PGI result was misleading, caused by inadequate trapping of the product that led to its redundant exchange with the medium. In the Glc-6-P to Fru-6-P direction in D2O we found a surprising overshoot of the equilibrium that was seen when the Fru-6-P was determined by a color test but not when determined by an enzymatic assay that went to completion (6). The colorimetric assay typically does not go to completion and therefore is sensitive to a kinetic deuterium isotope effect. The first Fru-6-P to be formed had derived some of its proton from the substrate as hydrogen. At later times this Fru-6-P acquired deuterium by back exchange with water and the recovered ester. However, an enzymatic reaction with a comparable mechanism would not be expected to show exchange because the reversal of the hydration step would remove the same oxygen that was introduced in forming the intermediate. Only if the intermediate was able to interchange the identical groups (positional exchange, not possible for C in this case) could one expect otherwise.

Middlefort and Rose (15) used positional isotope exchange to establish the formation of glutamyl-P as an intermediate in the glutamine synthetase reaction: glutamate + ATP + NH3 → glutamine + ADP + Pγ. A two-step mechanism, E + glutamate + ATP ↔ E-ADP-glutamyl-P ↔ E + glutamine + Pγ, was contraindicated by failure to observe ATP:ADP exchange unless NH3 was also present. However, this could have been due to tightly bound ADP. We realized that even tightly bound ADP might be able to achieve torsional equilibration of its phosphoryl oxygen, in which case the β-γ bridge O of ATP would scramble into non-bridge positions of reisolated ATP if ATP:ADP exchange proved to be the case. Indeed, positional isotope exchange was found in the absence of NH3 at a rate greater than required from the maximum rates of the forward and reverse net reactions (16).

Such is my confidence that all enzymatic reactions are stereospecific that finding one otherwise suggests that a nonenzymatic step must be part of the reaction sequence. As an example, the enzyme called methylglyoxal (MG) synthase actually produces enol-pyruvaldehyde (ePy), not MG, from dihydroxyacetone-P. The MG arises from
ketonization of the product ePy in solution. This was anticipated when the \(-\text{CH}_3\) group of the MG was found to have been made nonstereospecifically (17). The ketonization of ePy is a slow step for which no enzyme has been found. Instead, glutathione adds spontaneously to the carbonyl of the ePy, and the rapidly ketonized adduct becomes the substrate for reaction with glyoxalase I (18). Why the synthase did not evolve to carry out the ketonization step is a puzzle because there is a very active enzyme, glyoxalase III, that would utilize MG directly. Perhaps it has something to do with the fact that methylglyoxal is fairly toxic, forming stable complexes with proteins.


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